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## **Trait directed de novo population transcriptome dissects genetic regulation of a balanced polymorphism in phosphorus nutrition/arsenate tolerance in a wild grass *Holcus lanatus***

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1 Trait directed *de novo* population transcriptome dissects  
2 genetic regulation of a balanced polymorphism in phosphorus  
3 nutrition/arsenate tolerance in a wild grass *Holcus lanatus* L.

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## 1    **Summary**

- 2        • Here we characterize the genetic regulation of a single gene balanced  
3           polymorphism for phosphate fertilizer responsiveness, as well as arsenate  
4           tolerance, in wild grass *Holcus lanatus* L. genotypes screened from the same  
5           habitat, treated with high and low phosphorus (P) as phosphate.
- 6        • *De novo* transcriptome sequencing, RNAseq and SNP calling was conducted  
7           on extracted RNA. Roche 454 sequencing data was assembled into ~22,000  
8           isotigs and Illumina reads for phosphorous starved (P-) and phosphorous  
9           treated (P+) genovars of tolerant (T) and non-tolerant (N) phenotype were  
10          mapped to this reference transcriptome.
- 11       • Heatmaps of the gene expression data showed strong clustering of each  
12          P+/P- treated genovar, as well as clustering by N/T phenotype. Statistical  
13          analysis identified 87 isotigs to be significantly differentially expressed  
14          between N and T phenotypes and 258 between P+ and P- treated plants.
- 15       • This single gene for tolerance led to distinct phenotype transcriptomes, with  
16          large differences in post-translational and post-transcriptional regulation  
17          rather than in P nutrition transport and metabolism; and the identification of  
18          SNPs that systematically differ between phenotype, again in transcripts with  
19          predicted regulatory function including several proteases, kinases and  
20          ribonuclear RNA binding protein and concurrent up-regulation of expressed  
21          transposons in the T phenotype.

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## 1    **Introduction**

2    Grasses are known to have complex genomes of various size, often large, with  
3    extensive repetitive elements, local rearrangements, differences in genomic  
4    structure, ploidy level and chromosome number, and are consequently challenging  
5    with respect to whole genome sequencing and assembly (Buckler *et al.*, 2001;  
6    Feuillet & Keller, 2002; Jackson *et al.*, 2011, Hamilton & Buell, 2012). Transcriptome  
7    sequencing bypasses genomic complexity by focusing on protein coding genes  
8    (Hamilton & Buell, 2012) (~25,000 to 56,000 in grasses) derived from a well-  
9    conserved and relatively small proportion of the genome (~84% of gene families  
10    shared between grass sub-families) (The International Brachypodium Initiative,  
11    2010). As transcripts are sensitive to environment, RNA sequencing can be clustered  
12    by function through use of appropriate experimental manipulation to further aid in  
13    gene pathway identification (Suarez Rodriguez *et al.*, 2010; Urzica *et al.*, 2012;  
14    O'Rourke *et al.*, 2013).

15

16    While genomic sequencing has led to unparalleled advance in our understanding  
17    of the physiology and ecology of model organisms, it is not currently feasible  
18    (due to expense) to conduct trait led genome sequencing investigations in all  
19    wild species of interest (Nawy, 2012), rather there is a reliance in finding traits  
20    that exist (naturally or through mutation) in model species (Jackson *et al.*, 2011).  
21    While laboratory generated (site directed, knockdown, overexpression, chemical  
22    or radiation induced) mutants are essential for studying gene function and serve  
23    as breeding resource (Kuromori *et al.*, 2009), natural polymorphisms in non-  
24    model species are a greatly untapped resource.

1

2 The wild grass *Holcus lanatus* L., an outcrossing diploid ( $2n=14$ ) and closely related to  
3 *B. distachyon* (Aliscioni *et al.*, 2012), has a remarkable balanced polymorphism in  
4 arsenate tolerance, screened from a semi-natural, non-arsenic contaminated  
5 populations (Meharg *et al.*, 1993), coded by a single gene (Macnair *et al.*, 1992). As  
6 arsenate is a phosphate analogue it has been postulated that this polymorphism is  
7 maintained due to phosphorus nutrition, not arsenate tolerance *per se*, particularly  
8 as the tolerance gene co-segregates with suppression of High affinity Phosphate  
9 Transport (HAPT) (Meharg *et al.*, 1992a; Meharg & Macnair, 1992b), though an  
10 explicit ecological link to phosphorus status of soils has yet to be proven (Naylor *et*  
11 *al.*, 1996). This soil P responsiveness is addressed in this current study along with the  
12 transcriptomic consequences of being of tolerant (T) or non-tolerant (N) phenotype  
13 to ascertain why and how this polymorphism is maintained.

14

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17

## 1    **Materials and methods**

2    Chemicals used in experiments were Trace element grade or better, while all  
3    chemicals used for analytical purposes were Aristar grade.

4

### 5    ***Plant & soil collection***

6    Single tillers of *H. lanatus* were collected from a semi-natural grassland,  
7    Cruickshank Botanic Gardens (CBG), University of Aberdeen, UK, where *H.*  
8    *lanatus* is a dominant species. Only one tiller was taken from each of 250  
9    individual plants, and only isolated plants were selected, spaced at least 5 m  
10    from each other. Surface soil (0-10 cm) from the CBG population was collected  
11    and 2 mm sieved and stored at field moist conditions until use. Tillers were  
12    cultivated in a temperate glasshouse following potting (10 cm wide pots) into  
13    John Innes number 2 compost.

14

### 15    ***Plant growth characterization***

16    Tiller testing was conducted according to procedures outlined in Macnair *et al.*  
17    (1992). Following tiller tolerance testing, genotypes were selected for further  
18    study where their longest root length after 2 wk growth in arsenate free solution  
19    exceeded 100 mm. The plants were either classified as non-tolerant (N) or  
20    tolerant (T) based on their tolerance index (TI), where  $TI = 100 * (\text{root growth in}$   
21     $0.013 \text{ mM arsenate} / \text{root growth in absence of arsenic})$ , with this segregation  
22    shown in Fig. 1. Out of the 250 genotypes screened, ~30 of each phenotype were  
23    selected for detailed study.

24

1 A P fertilization experiment was conducted on these ~60 genotypes, split into N  
2 and T phenotype, where each genotype was a single replicate, and where the  
3 CBG soil was fertilized with phosphate (100 P mg/kg soil d.wt. as disodium  
4 phosphate). Plants were grown in control (no fertilization) and with fertilization  
5 for 60 d before harvesting. At harvest roots were washed free of soil and root  
6 and shoot dry weight recorded along with shoot P. P was analysed in  
7 powderised shoot by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS)  
8 on an Agilent 4000 instrument, following microwave assisted digestion (CEM-  
9 Technologies) in concentrated nitric acid.

10

#### 11 ***RNA preparation***

12 Tillers were grown hydroponically for 2 wk in 50 ml either complete Hoaglands  
13 solution, or that solution minus the phosphate, in individual centrifuge tubes with  
14 base of the tubes covered in tin foil to block out light. Plants were grown in a heated  
15 glasshouse (~18<sup>0</sup>C), under supplemental sodium lamps. Roche 454 sequencing was  
16 conducted on one non-tolerant replicate grown plus (P+) or minus (P-) phosphate. A  
17 genotype of the non-tolerant phenotype was chosen for 454 transcriptome  
18 sequencing and assembly as it was assumed that the non-tolerant phenotype would  
19 be the most P responsive from previous physiological studies (Meharg & Macnair,  
20 1992a). For Illumina sequencing a further 4 replicate individual genotypes (not  
21 including the genotype used for 454 sequencing), of each phenotype (T or N) were  
22 grown in P+ and P- in a factorial design, phenotype (tolerance) by phosphorous [P]  
23 treatment, *i.e.* 16 samples in total. The N genotype used for 454 sequencing, and  
24 another T genotype, where analysed by Illumina in the same experiment used for

1 454 sequencing, but where not included in the phenotype\*P Illumina analysis  
2 experiment analysis as there RNA was extracted at a different time point which lead  
3 to differential gene expression. However, these additional T and N genotype were  
4 used in SNP analysis so that SNP calling was conducted on 20 samples.

5

6 On harvesting blotted lightly dry roots and shoots for each replicate had their RNA  
7 extracted. Samples were ground under liquid nitrogen and total RNA was extracted  
8 using the Plant RNeasy extraction method (Qiagen), with the additional on-column  
9 DNase treatment. The resulting material was stored at -80°C until shipping on dry ice  
10 for analysis.

11

## 12 ***Transcriptome sequencing***

13 Sequencing was conducted at the Max Planck Plant Breeding Research Institute,  
14 Cologne, Germany. RNA was reverse transcribed to cDNA, fragmented, polyA  
15 enriched and sequenced on a 454 GS-FLX (Titanium Chemistry) and HiSeq 2000 (100  
16 bp paired end Illumina technology).

17

18 For generation of a reference transcriptome assembly, two normalized Roche 454  
19 libraries were prepared for one arsenic non-tolerant genovar (N) and sequenced on a  
20 Roche 454 GS-FLX using Titanium chemistry. Half a plate was used for transcriptome  
21 sequencing of phosphorous treated (N+) and half a plate for non-treated (N-) plants.

22

23 For gene expression and SNP analysis (RNAseq), a 100bp paired end Illumina  
24 sequencing library was generated for each of the 20 samples. These included all 4



1 samples from the first experiment (T-, N-, T+, N+), as well as another 16 samples  
2 (experiment 2) consisting of 4 tolerant (T) and 4 non-tolerant (N) genovars, receiving  
3 P- and P+ treatment (4 N-, 4 N+, 4 T-, 4 T+). Illumina reads were mapped to the 454  
4 reference transcriptome assembly.

5

6 For annotation of the reference transcriptome with standalone BLAST (blast-2.2.22,  
7 <ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/2.2.22/>), the  
8 Osativa\_193\_transcript and Osativa\_193\_peptide databases were downloaded from  
9 <ftp://ftp.plantgdb.org/download/Genomes/OsGDB/>, the plant-refseq database from  
10 <ftp://ftp.ncbi.nih.gov/refseq/release/plant/> and the nucleotide (nt) database from  
11 <ftp://ftp.ncbi.nih.gov/blast/db/>.

12

13 For generation of the reference transcriptome, the 454 reads were adapter and  
14 quality trimmed and assembled with Newbler version 2.6. After assembly, isotigs  
15 were annotated by blasting them against *Oryza sativa* transcript (BLASTn), *O. sativa*  
16 peptide, plant-refseq (BLASTx) and nt (BLASTn) using an e-value cutoff of 1.00 E-08.  
17 A BLAST report was compiled by parsing of all BLAST results with Perl scripts using  
18 BioPerl modules (Stajich *et al.*, 2002) to extract the top hit accession, description, E-  
19 value, percent identification (id) from each BLAST search. Annotation of putative  
20 function for *O. sativa*-peptides and *O. sativa*-transcripts were inserted into the Blast  
21 report after submission to [http://rice.plantbiology.msu.edu/downloads\\_gad.shtml](http://rice.plantbiology.msu.edu/downloads_gad.shtml)  
22 (annotation of all isotigs is reported in SI Annotated-DESeq-Result.xlsx).

23

24

1 For identification of differentially expressed transcripts/genes, 100bp paired end  
2 Illumina reads from experiment 1 (4 samples) and experiment2 (16 samples) were  
3 aligned to the assembled 454 reference transcriptome (isotigs) using bowtie  
4 (Langmead *et al.*, 2009), allowing multiple matches (option -a) reporting only the  
5 best hits obtained for each read pair (option --best --strata) with an allowed  
6 maximum of 3 end-to-end mismatches (option -v 3) to an isotig. For each of the 20  
7 samples the number of reported reads aligning to each isotig was counted with use  
8 of a Perl script.

9

10 Differentially expressed transcripts/isotigs were identified in R ([http://www.r-](http://www.r-project.org/)  
11 [project.org/](http://www.r-project.org/)) with package DESeq (Anders & Huber, 2010) using the FDR<0.1 as  
12 cutoff for significance. DESeq analysis was carried out on the Illumina data from the  
13 second experiment (n=4) for all 4 pairwise comparisons (N- vs. T-, N+ vs. T+, N- vs.  
14 N+, T- vs. T+) to allow identification of genes relevant to P+/ P- treatment and those  
15 involved in tolerant vs. non-tolerant phenotype. After DESeq analysis the log2 Fold  
16 Change (log2FC) of lowly expressed genes was recalculated as follows: DESeq  
17 calculated normalized counts of <5 were set to a baseline of 5. This allowed  
18 estimation of log2FC even if one of the treatments showed a mean normalized count  
19 of 0 and furthermore ensured that all 2-fold changes reported had normalized mean  
20 expression values of at least  $\geq 10$  in one of the treatments in question. Isotigs with  
21 False Discovery Rate (FDR) < 0.1 for any treatment comparison (344 isotigs) were  
22 submitted to BLAST2GO (default settings: BLASTx, nr, BLAST expect value 1.0E-03,  
23 number of BLAST hits 20) (Conesa & Götz, 2008) for further functional analysis

1 including Gene Ontology, Enzyme Code and Interpro domain search. The full  
2 annotated DESeq result is reported in SI Annotated-DESeq-Result.xlsx.  
3  
4 For identification of isotigs showing homology to specific proteins of interest  
5 proteins were blasted against all isotigs (tBLASTn). For visualisation of assigned  
6 protein homologies, some selected isotigs were translated into protein (expasy,  
7 <http://web.expasy.org/translate/>) and aligned with homologous plant protein  
8 sequences using mafft version7 (Katoh & Standley 2013). Mafft alignments were  
9 imported into Seaview (Gouy *et al.*, 2010), and the alignments exported (SI Figs. 1-8).  
10  
11 Samtools was used for identification of SNPs with variant and mapping quality >20  
12 (Li *et al.*, 2009). SNP tables of all 20 plants were merged and homozygous and  
13 heterozygous SNPs consistent across N and T phenotype (n=10) were extracted as  
14 potentially relevant drivers of N versus T phenotype using a Perl script.  
15  
16 The data is in the process of being submitted to Short Read Archive (SRA) at the  
17 European Nucleotide Archive ([www.ebi.ac.uk/ena/](http://www.ebi.ac.uk/ena/)), to be released upon  
18 publication.  
19  
20  
21  
22

## 1    **Results and Discussion**

2    There was a clear segregation into tolerant and non-tolerant classes (Fig. 1).

3    This was expected as previous studies have shown the ubiquity of this balanced  
4    polymorphism (Meharg *et al.* ,1993; Naylor *et al.*, 1996). When grown in their  
5    soil of origin the plants responded differentially to phosphate fertilizer (Fig. 2).

6    General Linear Modeling (GLM) (using Minitab (v.16)) of ranked data (due to  
7    non-normality of untransformed data) found a significant ( $P=0.004$ )

8    phenotype\*P fertilization interaction for shoot/root ratio, all other model terms  
9    were not significant for this comparison. This phenotype\*P fertilization

10   interaction term was caused by a decrease in shoot/root ratio, in tolerants

11   treated with P, showing that the tolerant phenotype is not reducing relative root  
12   growth in response to P nutrition. Plants normally reduce relative root

13   production under P nutrition (Gojon *et al.*, 2009). This is the first characterization

14   of a phosphate specific response for this polymorphism and the differential

15   allocation to root and shoot biomass in response to P availability likely to be the

16   reason why this polymorphism is maintained, and this will explained and

17   outlined in more detail in subsequent publications. There were no significantly

18   different (*i.e.*  $P>0.005$ ) terms for both shoot weight and shoot P, while only the

19   treatment term was significant for root weight. These data show that shoot P

20   (root P was not measured as it is impossible to remove all adhering soil which

21   greatly confounds interpretation) does not differ between phenotypes, and that

22   this shoot P status is not P fertilizer responsive, all indicating tight homeostasis,

23   a known characteristic with respect to plant P nutrition (Gojon *et al.*, 2009; Hill *et*  
24   *al.*, 2006).

25

1 Roche 454 sequencing generated ~1 million reads and a total of 474 megabases (MB)  
2 of sequence data for assembly of a reference transcriptome. *Circa*. 82% of all reads  
3 and ~85% of all bases (400MB) aligned. The inferred read error was 1.06%  
4 (Supporting Information [SI] Illumina-and-454-Stats.xlsx). Assembly with Newbler  
5 2.6 generated a reference transcriptome (SI 454Isotigs.fna) with a total of 22,313  
6 isotigs. The overall size of the assembled reference transcriptome was 29 megabases  
7 (MB). The average isotig size obtained was 1,302bp, the N50 Isotig size 1,489bp, the  
8 number of isotigs  $\geq 1$ kb was 12,828, (SI Illumina-and-454-Stats.xlsx, Sheet1). When  
9 BLASTed against *O. sativa*, plant refseq transcriptome databases and non-redundant  
10 nucleotide database (nt) (SI Annotated-DESeq-Result.xlsx)). Of these, 18,204  
11 returned a match against *O. sativa* transcripts, 18,954 returned a match against *O.*  
12 *sativa* peptides, 19,344 against plant refseq and 19,589 against nt (cutoff for  
13 significance  $< 1.00 \text{ E-}08$ ). BLAST against nt returned hits almost exclusively against  
14 plant cDNA/mRNA, predominantly *Hordeum vulgare* (barley), *Triticum aestivum*  
15 (wheat). Highest homologous matches against Plant-refseq were invariably identified  
16 against protein/transcript sequences of *Brachypodium*, *Sorghum bicolor* and *O.*  
17 *sativa* (rice), of these *Brachypodium* being the most frequent hit and most closely  
18 related to *H. lanatus* (Aliscioni *et al.*, 2012). Further statistics on assembly are  
19 reported in the SI Illumina-and-454-Stats.xlsx. The only previously reported *H.*  
20 *lanatus* gene sequence, AY704470, a CDC25 phosphatases homologue (Bleeker *et al.*,  
21 2006), was identified in all genotypes with isotig19077 showing 100% identity to this  
22 published sequence (tBLASTn) (SI Fig. 1), giving further verification of the  
23 transcriptome assembly.

24

1 Paired end Illumina sequencing produced an average of ~53 million reads for each of  
2 the 20 libraries giving a total of 1052 million 100bp paired end reads or 210  
3 gigabases (GB) of sequence data. Forty-eight percent (100GB) of the HiSeq data  
4 aligned successfully to the assembled 454 transcriptome without any prior clipping  
5 of reads, with an average of 25 million full length 100bp paired end reads (5GB) for  
6 each of the 20 libraries mapping successfully to isotigs with 3 or less end-to-end  
7 basepair mismatches (SI Illumina-and-454-Stats.xlsx). Of 22313 isotigs only 52 isotigs  
8 did not obtain any mapped paired end Illumina reads when aligning all Illumina reads  
9 from (all 20 individual samples) giving good verification of the 454 reference  
10 transcriptome assembly (SI RawCounts-per-isotig.xlsx). The isotigs, which did not  
11 obtain any mapped Illumina reads may either have been too short (less than 250bp)  
12 or misassembled. Non-aligned Illumina reads on the other hand are likely to be a  
13 mixture of poor quality reads, reads containing adapter sequence, which would  
14 require end-trimming, or alternatively originate from lowly expressed transcripts,  
15 which may not be represented in the 454 reference library, but may still be picked  
16 up amongst the Illumina sequences due to the much deeper coverage achieved with  
17 Illumina technology. Transcript expression result showed strong clustering of each  
18 P+ and P- treated genotype verifying that each plant was indeed a different  
19 genotype (Fig. 3). T genotypes clustered together, as did N, showing that the  
20 phenotypes had distinctive transcript expression signatures. Differential expression  
21 analysis was carried out with DESeq (Anders & Huber, 2010) and identified 344  
22 isotigs, with a False Discovery Rate (FDR) of 0.1, significantly up or down-regulated in  
23 response to either P+/P- treatment or N/T phenotype (Fig. 4, SI Annotated-DESeq-  
24 Result.xlsx). Of these 87 isotigs were shown to be differentially expressed between N

1 and T phenotype, while the majority, 258 isotigs, were shown to be differentially  
2 expressed in response to different P $\pm$  nutrition treatment. There was no overlap  
3 between the significant isotigs identified for P+/- and N/T phenotype response.  
4 There are a number of potential explanations for the independence of transcripts  
5 between N/T and P+/P- nutrition. The first is that a single regulatory gene, given that  
6 we know that arsenate tolerance is under single gene control (Macnair *et al.*, 1992),  
7 is controlling transcript production of a host of genes in the T/N comparison. The  
8 second, which is not exclusive of the first, is that differences in metabolism resulting  
9 from differential function of a gene(s) may lead to feedback regulating transcripts of  
10 interrelated functions such as the obvious impact of P starvation on P metabolism  
11 observed here (Figs. 3 and 4). Differences in P stress perception by plants are known  
12 to induce a host of differential responses, such as tillering, root biomass production,  
13 arbuscular mycorrhizal regulation, and rhizosphere excretion of dicarboxylic acids to  
14 mobilize phosphate from iron minerals, and this will lead to differential regulation of  
15 a network of genes (Chiou *et al.*, 2011; Gojon *et al.*, 2009; Hill *et al.*, 2006). Again, the  
16 arsenate tolerance gene has a range of pleiotropic consequences (shoot/root  
17 biomass, HAPT suppression and arsenate tolerance itself), fitting well such a  
18 feedback and/or upstream regulator model.

19

20 The amplitude of fold-change was greater between phenotypes than between P  
21 treatments, while annotation was better for P responsive genes (Fig. 4). Annotated  
22 genes in classes that are highly relevant to the current study show that transcripts  
23 significantly differentially regulated between T and N phenotype are dominated by  
24 kinases, pathogen resistance, plant growth regulators (PGR), proteases, transposable

1 elements (TEs) and RNA directed activity, but none involved in phosphate transport  
2 (Annotated-DESeq-Results.xlsx, Fig. 4). It has previously been shown that one  
3 consequence of having the arsenate tolerance gene is suppression of high affinity  
4 phosphate/arsenate transport (Meharg *et al.*, 1992a,b). The results presented here  
5 indicate, therefore, that post-transcriptional and post-translational mechanisms play  
6 a key role in this suppression, as there was no differential expression of transcripts  
7 involved in phosphate transport between phenotype.

8

9 With respect to T phenotype the only annotated gene absent compared to N, where  
10 it is highly expressed, is a kinase receptor (isotig09647). Furthermore, isotigs with  
11 significant homology to cbl-interacting kinase, MAPK kinase and serine/threonine  
12 kinases had systematic differences in SNPs between N and T phenotype as did isotigs  
13 showing homology to proteasome associated protein, transferases and a  
14 ribonucloprotein/RNA recognition protein (Fig. 5, SI Table 1).

15

16 A gene that codes for rice adaption to soil P stress, PSTOL1, has been characterized  
17 (Gamuyao *et al.*, 2012). This gene is an enhancer of early root growth and over  
18 expression leads to increased grain yields, hypothesized to be due to more efficient P  
19 capture due to larger root systems, with larger root systems characterizing the *H.*  
20 *lanatus* T phenotype here (Fig. 2). This gene is a kinase and some homologous  
21 sequences were identified in *H. lanatus*, such as isotig20112 which showed ~ 88%  
22 (identities 145/164) homology to the serine/threonine protein kinase  
23 LOC\_Os01g04570.2 and 63% (identities 108/169) to Pstol1/OsPupK46-2, also  
24 annotated as serine/threonine protein kinase (SI Fig. 2) and isotig03216, which



1 showed 50% (identities 158/312) to protein serine/threonine kinase  
2 LOC\_Os01g04570.2 and 49% (identities 154/313) to Pstol1/OsPupK46-2. Isotig20112  
3 was expressed in all T and N phenotypes but was ~4 fold down-regulated in 2 out of  
4 4 N phenotypes, with no P effect. isotig03216 was again expressed in all N and T  
5 phenotypes, but N5, N4, N2 as well as T4 showed ~>3 fold lower expression  
6 compared to T2, T3, T5, N3, but the overall observed ~2 fold up-regulation in T-  
7 versus N- was not statistically significant (SI AnnotatedDeseqResult.xlsx).

8

9 Other transcripts belonging kinases, some of these showing homology to cbl-  
10 interacting kinases 9, 14 & 23, were found to be up-regulated under low P status  
11 (Fig. 4, SI AnnotatedDeseqResult.xlsx). Cbl-interacting kinases are serine/threonine  
12 protein kinases, as is PSTOL1 (Gamuyao *et al.*, 2012). The only transcript absent in T  
13 compared to N phenotype (present in 3 out of 5 N phenotypes and absent in all 5 T  
14 phenotypes), isotig09647, noting that there was also a transposon severely  
15 suppressed, was a receptor protein kinase. Another probable serine/threonine  
16 protein kinase WNK2-like, isotig18018, was ~60 fold up-regulated in T compared to  
17 N. It is apparent that kinases play a role in both T/N phenotypic and P responsive  
18 differences in the *H. lanatus* transcriptomes presented here and that there is  
19 possibly some level functional redundancy. It is pertinent in this context that some  
20 kinases have recently been identified as being central in phenotypic differences in  
21 plant root response to P status (Gamuyao *et al.*, 2012) as well as shown to be up  
22 regulated in response to arsenate stress (Huang *et al.*, 2012).

23

1 Also of note is that an auxin binding protein (isotig16840) was highly expressed in  
2 the T phenotype compared to the N phenotype. The alignment of the translated  
3 isotig16840 is shown in SI Fig. 3 and shows a high homology to auxin binding rice  
4 and *Brachypodium* proteins. One of the key differences between T and N phenotype  
5 identified here was in root biomass (Fig. 2). Auxins, and associated expansins, have a  
6 key role in root growth (Cosgrove, 1999). The higher expression of an auxin-binding  
7 protein with decreased expression of an expansin precursor transcript in T  
8 phenotype, compared to N, and the enhanced expression of an auxin responsive  
9 protein (isotig11028) under high P (SI AnnotatedDeseqResult.xlsx) suggest that auxin  
10 signaling is central to the difference in root biomass regulation between T and N. The  
11 alignment of translated isotig12721 with expansin precursor can be seen in SI Fig. 4.

12

13 While most expressed transposons, some of these annotated with RNA directed  
14 activity, were up regulated in T compared to N (isotig13038, 13187, 13075, 15546,  
15 07417), one transposon of the en spm subclass (isotig02887/02888) and one  
16 unclassified nucleic acid binding transposon (isotig05913) were suppressed in T  
17 compared to N, (SI AnnotatedDeseqResult.xlsx). Transposons and retrotransposons,  
18 the most frequent and strongly differentially expressed class of isotigs between  
19 phenotypes, are thought to play a role in post-transcriptional regulation with  
20 silencing of TEs involving both transcriptional and post-transcriptional mechanisms  
21 (Mirouze & Paszkowski, 2011; Okamoto & Hirochika, 2001). In plants,  
22 retrotransposons are commonly known to be expressed under conditions of stress  
23 (Grandbastien, 1998). It is also thought that retrotransposon activation is sensitive to  
24 environment (Mirouze & Paszkowski, 2011; Grandbastien, 1998), further enhancing

1 their candidacy for regulating stress responses, such as nutritional deficiencies.  
2 Transposable Elements (TEs) can evoke gene variation and functional changes (Gao  
3 *et al.*, 2012) and are a source of small RNAs and implicated in gene-regulation in  
4 both animals and plants (McCue & Slotkin, 2012). A role for small RNAs in regulation  
5 of P starvation is emerging (Fang *et al.*, 2009; Hsieh *et al.*, 2009; Chiou & Lin, 2011;  
6 O'Rourke *et al.*, 2013). Further to this, a potential role of a small RNA targeting  
7 transcripts involved in posttranscriptional/posttranslational regulation leading to T  
8 and N phenotype in *H. lanatus* is worth further investigation.

9

10 A suite of isotigs involved in protein degradation including two with homology to  
11 aspartic proteinase nepenthesin precursor (isotig17128, isotig10719), one FtsH  
12 protease (03772) and a ubiquitination like protein (isotig20448) were suppressed in  
13 T compared to N phenotype, indicating that post-translational protein degradation  
14 may be an important factor for the N phenotype. With respect to P treatment,  
15 ubiquitin-domain protein (isotig17019), protease inhibitor (isotig19161), putative  
16 subtilisin homologue, a non-specific protease (isotig03645) were significantly up  
17 regulated in response to P starvation, while ubiquitin conjugating enzyme like  
18 (isotig08177), ICE-like protease p20 domain containing protein (isotig10848),  
19 putative Deg protease homologue (isotig09222) and LTPL113 - Protease inhibitor  
20 (isotig19161) were significantly down regulated in response to P starvation.

21

22 Furthermore, 6 consistent homozygous and 30 consistent heterozygous SNPs for N  
23 versus T phenotype (n=10) were identified (Fig. 5). These occurred predominantly in  
24 transcripts associated with regulatory function such as proteolysis (protease,

1 proteasome subunit, heat shock), protein modification (cysteine desulferase,  
2 glycosyl transferase, transferase, kinase) and RNA recognition (RNA-binding,  
3 ribonucleoprotein) and were a mixture of transitions and transversion SNPs (Fig. 5).  
4 Ubiquitins, which mark proteins for proteasome mediated degradation are thought  
5 to have a key role in regulation of plant SPX domains in response to P stress (Wu *et*  
6 *al.*, 2012), so it is particularly interesting that we find that the largest number of  
7 SNPs (3 transversion and 1 transition SNP) between T and N in an putative  
8 proteasome subunit (isotig11038) as well as SNPs in FtsH protease (isotig03772) and  
9 heat shock protein (isotig03795/03796). Also while we assume that these SNPs are  
10 of genomic origin, without the genomic sequence of *H. lanatus* it is not possible to  
11 rule out that targeted mRNA editing may be involved in some of these cases. RNA-  
12 editing, which has first been identified in the cox2 mRNA of *Trypanosoma brucei*, is  
13 thought to play an important role in organelles (plastids and mitochondria) of plants,  
14 with those identified typically involving a change of specific C to U, but other  
15 changes can as yet not be ruled out (Grennan, 2011; Jiang *et al.*, 2012). The role of  
16 RNA-editing in plant plastid as well as nuclear encoded RNA/mRNA, remains to be  
17 further investigated by systematic sequencing of plant genome (DNA) and  
18 transcriptome (cDNA) as has been described for identification of RNA editing sites in  
19 human studies (Ramaswami *et al.*, 2012). While RNA-binding proteins of the  
20 pentatricopeptide repeat family, multiple organellar RNA editing factor and  
21 chloroplast ribonucleoproteins are known to be involved, further proteins remain to  
22 be identified (Tillich *et al.*, 2009; Grennan, 2011; Takenaka *et al.*, 2012). So it is  
23 noteworthy in this context that one of the homozygous SNPs identified between T  
24 versus N phenotype in this study is in isotig05374 which shows 70% homology to a

1 RNA recognition motif containing protein/predicted ribonucleoprotein. Further to  
2 that, exonuclease (isotig08248), which mediates RNA degradation (Stoppel *et al.*,  
3 2012), is strongly up regulated in the N phenotype. Thus both the N/T gene  
4 expression result as well as the SNPs obtained for N/T phenotype suggest that post-  
5 translational regulation of proteins via the ubiquitin-proteasome system plays an  
6 important role in determining the N and T phenotype and furthermore points to a  
7 potential role of post-transcriptional regulation (RNA degradation and a possible role  
8 of RNA editing). A causative upstream master regulatory gene, inducing post-  
9 translational and maybe also post-transcriptional events of consequence for  
10 arsenate resistance and P uptake efficiency in these plants remains to be identified  
11 and potential involvement of small RNAs should be investigated in this context.

12

13 The transcripts differentially regulated by P treatment were more completely  
14 annotated compared to between phenotype (SI AnnotatedDeseqResult.xlsx), with  
15 many of the genes identified as being significant having well known roles in P  
16 transport and metabolism, as well as those involved in post-transcription, post-  
17 translation, and signaling (Fig. 4, SI Annotated-DESeq-Results.xlsx). Some gene  
18 expression responses involved phosphate transport. These include 3 isotigs  
19 annotated as phosphate co-transporters and 2 with SPX domains. All showed the  
20 same general pattern, with respect to transcript counts, with up-regulation under P  
21 starvation, and down-regulation in P replete treatment, but there was little  
22 difference with respect to the expression changes in response to P-/P+ in the T and N  
23 phenotype. Alignments of phosphate transport translated isotigs to proteins are  
24 shown in SI Fig. 5 & 6. Isotigs01092, 18981, 09507 showed strong homology to

1 AAM49810.1 a putative rice HAPT (SI Fig. 5), and isotig16690 to Q651J5 phosphate  
2 transporter PHO1-3 known to be induced under P deficient conditions (SI Fig. 6). Of  
3 the few isotigs with significantly higher counts in P+ compared to P- in T, but not in  
4 N, was an auxin induced protein (isotigs11028). Given the strong induction in an  
5 auxin-binding transcript (isotig16840) in the T versus N phenotype and the observed  
6 differences in root growth, auxin mediated response appears to be an important  
7 differentiator of the T/N phenotype. The rice and *Brachypodium* matches to the  
8 translated isotig16840 protein sequence are shown in SI Fig. 3.

9

10 A number of recent studies have found up-stream regulators of low phosphorus  
11 adaption responses in plants with ALFIN—LIKE proteins implicated in regulating root  
12 hair growth in Arabidopsis under P stress (Chandrika *et al.*, 2013). ALFIN-LIKE  
13 proteins are a small family of plant Homeo Domain (PHD) containing putative  
14 transcription factors with a methylated histone residue binding component and  
15 ALFIN-LIKE 6 was shown to control the transcription of a range of genes involved in  
16 growth in particular root hair growth (Chandrika *et al.*, 2013). A homologue of this  
17 gene was identified in our *H. lanatus* transcriptome (isotig02053) and the translated  
18 isotig showed high similarity to *A. thaliana* and even higher similarity to *B.*  
19 *distachyon* ALFIN-LIKE protein (SI Fig. 8), providing further evidence of the overall  
20 quality of the 454 transcriptome assembly, though this transcript was equally highly  
21 expressed in all N and T plants and not differentially regulated between either P  
22 treatment or phenotype.

23

1 Many of the transcripts responsive to P nutrition are regulated as expected, with  
2 many obviously involved in P nutrition and generally strongly up regulated in  
3 response to P stress. For example the 4 phosphate transport related transcripts, and  
4 the 2 SPX transcripts, identified in *H. lanatus* in this study were induced by P stress,  
5 in line with what has been observed generically in literature studies, as exemplified  
6 in the phosphate responsive transcriptome of white lupin (O'Rourke *et al.*, 2013).  
7 Other transcripts coding for proteins involved in P regulation or transport identified  
8 (SI Annotated-DESeq-Result.xlsx) include glycerophosphodiester/phosphodiesterase,  
9 sucrose-phosphate synthase, glucose pyrophosphorylase, purple acid phosphatase,  
10 phosphatase, glucose-6-phosphate phosphate translocator, for example, also  
11 differentially regulated when comparing P stressed and P repleat lupin (O'Rourke *et*  
12 *al.*, 2013).

13

14 With respect to arsenate tolerance, the character used to screen the phenotype  
15 understudy, one gene with a putative role in arsenic transport/metabolism, besides  
16 phosphate transporters and their regulators, was an arsB-like gene. The alignment of  
17 isotig09604 with ars-B like protein can be seen in SI Fig. 9. ArsB, is widely present in  
18 arsenic resistant bacteria where its role is as an arsenite efflux channel (Yang *et al.*,  
19 2012), though in plants these class of aqua-glycerin prions also are involved in silicic  
20 acid transport (Zhao *et al.*, 2009). This gene (isotig09604) was more strongly  
21 expressed in T compared to N phenotype as was aquaporin Pip1-5 like  
22 (Plasmamembrane Intrinsic Protein) (isotig00796/00797/00798), but this is only  
23 involved in transport of water and small neutral solutes. Logoteta *et al.* (2009) found  
24 that differential efflux of arsenite by *H. lanatus* was not found between T and N

1 phenotype. A protein, HLASR, has been identified in *H. lanatus* to have a  
2 constitutive, but not an adaptive role, in metabolism of arsenate, namely as an  
3 arsenate reductase (Bleeker *et al.* 2006). It was thought that this HLASR gene only  
4 had a secondary role with respect to arsenic metabolism and that its primary role  
5 was homologous to CDC25 phosphatases, which activate cycline-dependent kinases  
6 in *A. thaliana*, which are involved in cell cycle regulation. HLASR is also thought to  
7 have a role in GSH oxidation (Bleeker *et al.*, 2006). An exact protein match  
8 (isotig19077) to this Cdc25-like *H. lanatus* ASR, the only gene sequence previously  
9 published for this species, was found in all 10 N and 10 T transcriptomes (SI Fig. 3),  
10 and was shown not to be differentially regulated, confirming that it has no adaptive  
11 role in tolerance.

12

13 Returning to the fact that the trait under study here is due to a single *loci* and that it  
14 leads to a range of distinct phenotypic traits is indicative that an upstream regulatory  
15 gene is involved. It is now well established that P responsive genes a) give rise to  
16 transcriptome signal cascades and/or b) are involved in these signal cascades (*Wu et*  
17 *al.* 2013; Chiou & Lin, 2011). We identified 87 transcripts whose transcript  
18 expression significantly differed between T and P phenotype, and 19 transcripts (17  
19 with functional annotation) with consistent SNPs (36 SNPs in total) in all 10 T versus  
20 N genotypes (SI Table 1). It is noteworthy that consistent SNPs and significant gene  
21 expression changes between N versus T phenotypes are aggregated in transcripts  
22 with regulatory functions (Fig.s 4 and 5). Differential post-translational and/or post-  
23 transcriptional regulation (involving ubiquitin, proteases, kinases, methylation,  
24 transposons, retrotransposons and RNA binding proteins) of HAPT or proteins



1 acting on HAPT, therefore, appear to define the N/T phenotype. Whether identified  
2 SNPs are all genomic SNPs or whether in some cases mRNA editing may be involved  
3 remains to be elucidated. A master regulatory gene, potentially to be found within  
4 our list of target genes or possibly in form of an as yet to be identified small RNA,  
5 leading to the observed effect on genes involved in post-translational events  
6 including protein degradation via the ubiquitin/proteasome complex and potentially  
7 also post-transcriptional events mediated by RNA binding proteins, is as yet to be  
8 identified. This characterization of the genetic consequences the P response  
9 polymorphism in *H. lanatus* provides an unparalleled insight into the signal cascades,  
10 optimized under natural selection, involved in P nutrition and has major  
11 consequences for understanding how plants respond to phosphorus nutrition and  
12 adaptation to arsenate in their environment. We anticipate that this as yet unknown  
13 master regulatory gene and it's downstream targets, which we have already  
14 identified, will be of significant consequence for future study and breeding of P-  
15 efficient forage plants and cereal crops.

16

17

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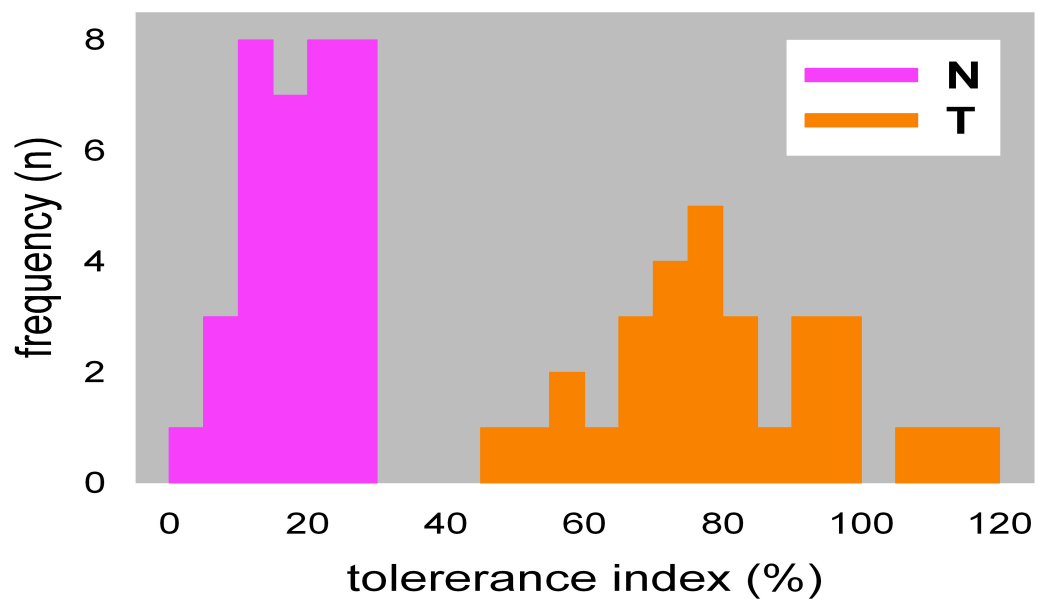
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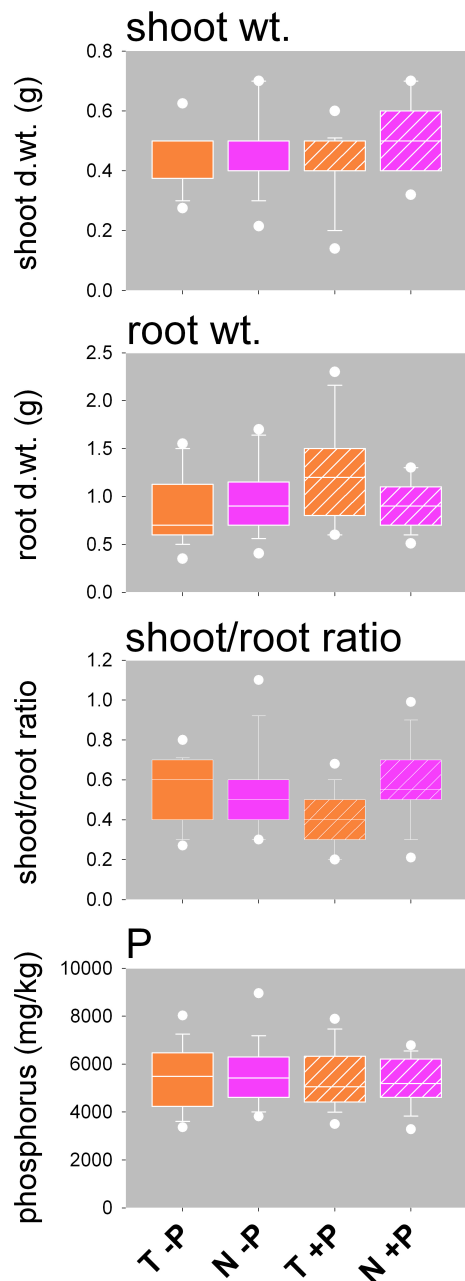
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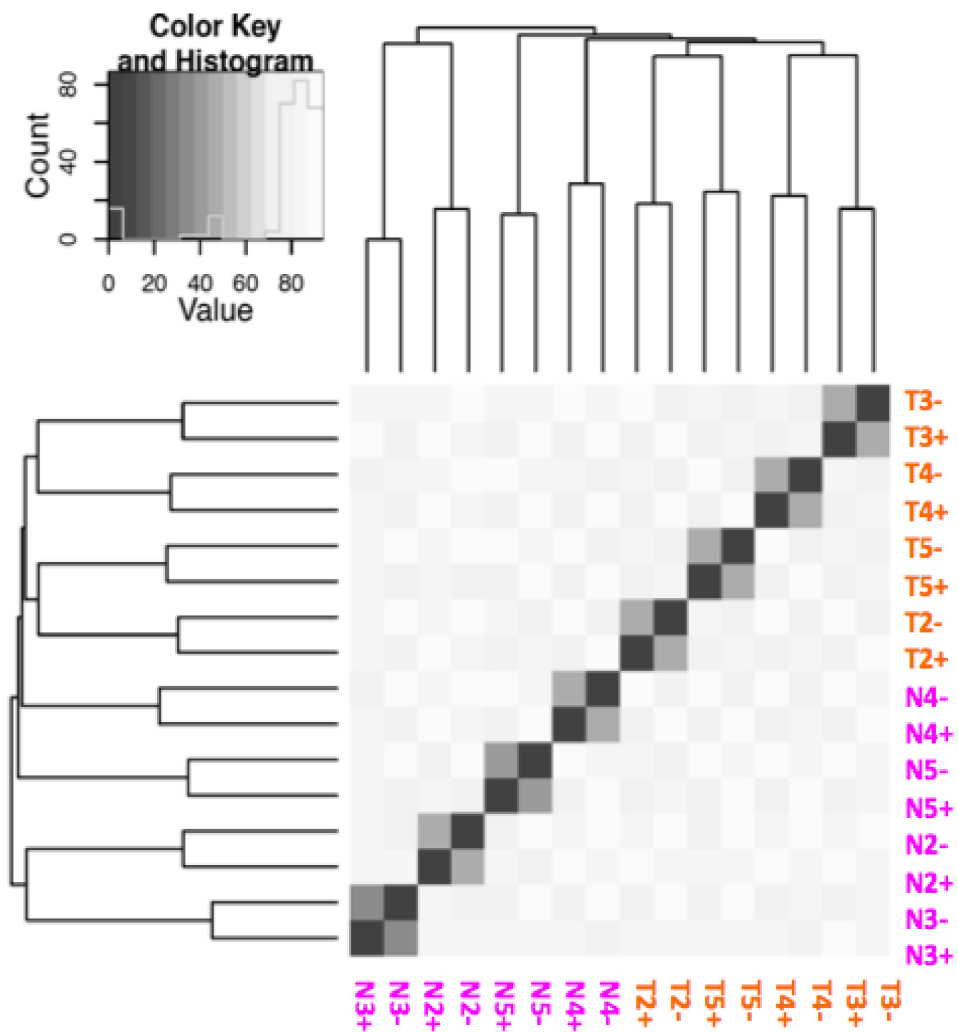
4 **Figure 1.** Tolerance index ( $100 \times \text{root length in +As} / \text{root length in -As}$ ) of Cruickshank  
5 garden *H. lanatus* population grown hydroponically in 0.013 mM arsenic as arsenate  
6 for 2 wk..

7

8



**Figure 2.** Box plots of Cruickshank *H. lanatus* response to phosphate fertilization (100 mg/kg P on a soil d.wt. basis) when grown from tillers for 2 mth. on their soil of origin. Arsenate tolerant plants are orange bars, non-tolerant pink bars. No P fertilization is without shading while P fertilized plants are shaded. Shown on the bars are the median, 25<sup>th</sup> & 75<sup>th</sup> (outer box), 10<sup>th</sup> and 90<sup>th</sup> (whiskers) and 5<sup>th</sup> and 95<sup>th</sup> (dots) percentiles.



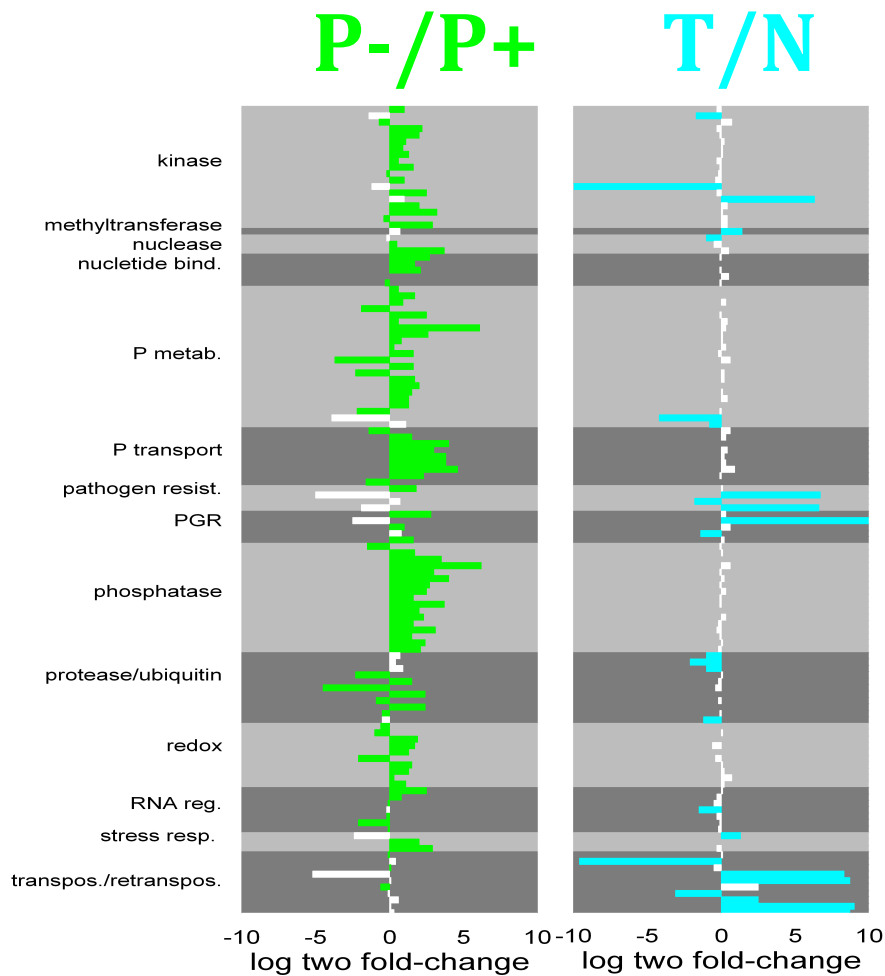
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5 **Figure 3:** Heatmap of the RNAseq gene expression result showing clustering of P+/-  
6 of each genotype as well as T and N phenotype



**Figure 4.** RNAseq gene expression result: up and down regulation identified for P-/P+ and T/N. As there was no statistical difference between phosphorus treatments and tolerance, the average of all treatments by either P treatment or by phenotype was used in this analysis. All genes significantly differentially regulated (at FDR = 0.01) in either treatment (P or phenotype) are shown. Where the ratio was significant between treatments the bars are coloured, where not significant, the bars are white.

		T		N	
		isotig position			
protease	3772	1240	C	T	
		616	C	T	
		1249	T	C	
protease	877	954	R (A/G)	A	
proteasome subunit	11038	104	R (A/G)	A	
		107	K (G/T)	T	
		659	W (A/T)	T	
		688	M (C/A)	C	
kinase, MAPK	8620	2482	S (C/G)	C	
		2485	Y (C/T)	T	
kinase, cycl. Dep.	13949	862	R (A/G)	A	
		868	S (C/G)	G	
kinase, serine/threonine	16533	109	Y (C/T)	T	
		40	Y (C/T)	T	
cysteine desulfur.	10743	112	R (A/G)	G	
cyt. P450	1775	281	T	C	
glycosyl transfer.	13914	969	S (C/G)	C	
heat shock	3795	1184	R (A/G)	G	
		878	M (C/A)	C	
ketol-acid reductosiom.	4035	1655	S (C/G)	C	
phosphoglycer. dehydrog.	9180	1930	K (G/T)	G	
RNA recog.	5374	460	T	C	
transferase	10716	769	Y (C/T)	C	
Zn-finger	11163	1068	Y (C/T)	C	

**Figure 5.** Consistent T versus N (n=10) homozygous (~100%) and heterozygous (~50%) SNPs identified are found predominantly in transcripts with regulatory function. In red transversions A <-> C, A <-> T, G <-> C, G <-> T (interchange of two ring purine (AG) for one ring pyrimidine (CT)). In green the more common transitions A<->G, C<->T (interchange of two ring purines (AG) or one ring pyrimidines (CT)).